

Diagnosis of a person's risk of developing alcoholism.

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FIELD OF THE INVENTION

This invention relates to methods for diagnosing a person's susceptibility for
10 having a risk for the development of alcoholism. The invention relates further
to methods for preventing or treating persons diagnosed for having risk for
the development of alcoholism, in order to prevent the development of said
condition. The invention also concerns methods to investigate or screen
pharmaceuticals or genetic aims useful in the prevention or treatment of
15 alcoholism, by using an animal model including a transgenic animal.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background
20 of the invention, and in particular, cases to provide additional details
respecting the practice, are incorporated by reference.

Neuropeptide Y (NPY) is a hexatriocontapeptide amide that is well
characterized as a neuromodulator in the central nervous system [Gray and
25 Morley, 1986; Lundberg et al., 1982]. The best known effects of NPY are
stimulation of feeding [Clark et al., 1985; Levine and Morley, 1985; Stanley
and Leibowitz 1985] and increased energy storage through lipoprotein lipase
activation in white adipose tissue [Billington et al., 1991; Billington et al.,
1994]. Recent findings in rodents suggest that NPY may also be a potential
30 regulator of ethanol consumption [Ehlers et al., 1998a; Ehlers et al., 1998b;

Thiele et al., 1998; Cokerill, 1998; Tecott and Heberlein, 1998]. The preference for alcohol seems to be inversely related to NPY levels in brain [Thiele et al., 1998]. NPY-deficient mice show increased consumption of ethanol, whereas transgenic mice that overexpress a NPY gene have a lower
5 preference for ethanol and are more sensitive to its sedative/hypnotic effects [Thiele et al., 1998]. NPY and ethanol have a similar electrophysiological profile [Ehlers et al., 1998b], and both are known to have anxiolytic properties [Thiele et al., 1998; Heilig et al., 1992; Palmiter et al., 1998; Stewart et al., 1993]. In addition, NPY might influence consumption
10 behaviors through reward effects [Ehlers et al., 1998a; Tecott and Heberlein, 1998]. NPY is expressed in the amygdala and nucleus accumbens, structures of the mesolimbic dopamine system that are thought to mediate the rewarding aspects of food, alcohol and certain drugs [Tecott and Heberlein, 1998; Ault et al., 1993; Jewett et al., 1992]. Despite the circumstantial evidence from
15 animal models, no studies on the role of NPY in the regulation of alcohol consumption in humans have yet been published.

A novel finding of a common polymorphism in the signal peptide of NPY was recently reported [Karvonen et al., 1998]. After screening the entire
20 coding region of the NPY gene for sequence variants, a thymidine(1128) to cytosine(1128) polymorphism(T1128C) was identified, resulting in a substitution of Leu(7) to Pro(7) in the signal peptide part of preproNPY. The Pro (7) in NPY showed a strong association with elevated serum cholesterol levels [Karvonen et al., 1998].

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In the present study we found that the Leu (7) to Pro (7) polymorphism in NPY is related to the level of alcohol consumption in an unselected male population sample from the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD) [Salonen, 1988; Lakka et al., 1994].

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Figure 1c shows the nucleotide sequence of the human preproNPY mRNA (SEQ ID NO:5, with the protein sequence set forth in SEQ ID NO:6). The arrow shows the position in which thymidine (t) of the normal mRNA is replaced by cytosine (c) to give the mutant mRNA.

The determination can be carried out either as a DNA analyse according to well known methods, which include direct DNA sequencing of the normal and mutated NPY gene, allele specific amplification using the polymerase chain reaction (PCR) enabling detection of either normal or mutated NPY

sequence, or by indirect detection of the normal or mutated NPY gene by various molecular biology methods including e.g. PCR- single stranded conformation polymorphism (SSCP)-method or denaturing gradient gel electrophoresis (DGGE). Determination of the normal or mutated NPY gene
5 can also be done by using restriction fragment length polymorphism (RFLP)-method, which is particularly suitable for genotyping large number of samples.

The determination can also be carried out at the level of RNA by analysing
10 RNA expressed at tissue level using various methods. Allele specific probes can be designed for hybridization. Hybridization can be done e.g. using Northern blot, RNase protection assay or in situ hybridization methods. RNA derived from the normal or mutated NPY gene can also be analysed by converting tissue RNA first to cDNA and thereafter amplifying cDNA by an
15 allele specific PCR-method and carrying out the analysis as for genomic DNA as mentioned above.

Alternatively, the determination can be carried out as an immunoassay where a sample is contacted with an antibody capable of binding the signal peptide
20 or said peptide associated with any other cleavage product of preproNPY.

Antibodies can be raised against normal or mutated preproNPY or more specifically against normal or mutated signal peptide part of the NPY. The production of antibodies can be done in experimental animals in vivo to
25 obtain polyclonal antibodies or in vitro using cell lines to obtain monoclonal antibodies.

A person diagnosed for having a risk for the development of alcoholism can be treated for the prevention of developing said condition by administering to
30 said person an effective amount of an agent counteracting the influence of the

mutated NPY gene. This can be done by specific gene therapy aimed to repair the mutated NPY sequence, or by administering pharmacotherapies, which are aimed to modulate synthesis, release or metabolism of the endogenous NPY, or to interact in a specific manner at NPY target sites by modulating effects of NPY with specific NPY receptor proteins. Currently, five different subtypes of NPY receptors have been cloned and characterized (Y1-Y5 receptors) and drug molecules specifically interacting with these NPY receptors have been synthesized. The pharmacotherapy described is not limited to only these named receptors or mechanisms, but also covers other NPY receptors and related mechanisms to be discovered including the secretion of NPY.

The influence of the mutated NPY gene in a patient can be counteracted by using an antisense therapy or gene switching or replacement, which includes targeted correction of disease-related mutation or site-directed inactivation of the mutant allele by homologous recombination.

The antisense therapy refers to methods designed to impair translation through direct interactions with target messenger RNA (mRNA). This can be accomplished by applying a targeted oligonucleotide, which forms Watson-Crick base pairs with the messenger RNA whose function is to be disrupted. The inhibition of gene expression by antisense oligonucleotide depends on the ability of an antisense oligonucleotide to bind a complementary mRNA sequence and prevent the translation of the mRNA. It is possible to correct a single mutant base in a gene by using an oligonucleotide based strategy (Giles et al., 1995; Schwab et al., 1994; Yoon et al., 1996). A short, 7 or 8 bases, oligonucleotide is enough to possess an antisense activity and specificity, which depends greatly on the flanking sequences of the target RNA. Binding should be enough to promote stable binding and RNase H – mediated cleavage.

The influence of the mutated NPY gene is preferably counteracted by using a short, allele specific oligonucleotide, which includes the sequence of mutated part: ...cga ct/cg ggg.... This can be accomplished by using oligonucleotides of various lengths, but all recognizing the mutated base sequence. According to the predicted secondary structure of the preproNPY mRNAs (Schemes 1 and 2), the best target sequence is between -9 and +2 bases around the mutation i.e. sequence targeting to 3'-ac aag cga ctg g-5'. This sequence contains 'bulbs' which are known to enhance the binding of oligonucleotide to the target mRNA.

It is possible to use unmodified oligonucleotides, but to increase their stability, nuclease resistance, and penetration to the nucleus, several modifications of oligonucleotide can be used. A relatively large number of modified pyrimidines have been synthesized, mainly C-2, C-4, C-5, and C-6 sites, and incorporated into nucleotides. Also purine analogs can be synthesized and incorporated into oligonucleotides. The 2' position of the sugar moiety, pentofuranose ring, is substituted with methoxy, propoxy, O-alkoxy or methoxyethoxy groups. A new backbone for oligonucleotides that replace the phosphate or the sugar-phosphate unit has been made, like C-5 propynylpyrimidine-modified phosphothioate oligonucleotides. Also chimeric oligonucleotides with 5'- and 3'-ends are modified with internucleotide linkages, like methylphosphorothioate, phosphodiester, or methylphosphonate can be used. A relatively new technique is conformationally restricted LNA (locked nucleic acid) oligonucleotides and peptide nucleic acids. Bioengineered ribozymes are structurally different, but their specificity also relay on the recognition of the targeted mRNA sequence.

Gene replacement or gene switching techniques inactivate the mutated gene sequence and introduce a corrected one. This can be accomplished by

transfecting exogenous gene with normal coding sequence and blocking mutant coding sequence with antisense oligonucleotide. Also a technique with only introducing a corrected normal sequence without disrupting the mutated sequence could be use. This could be used in heterozygous cells i.e.

5 cell carrying one normal allele and one mutated allele resulting in an overexpression of normal alleles. Also homozygous mutant cells could be treated resulting in a dominant positive –effect i.e. the normal allele is expressed in higher degree than the mutant allele.

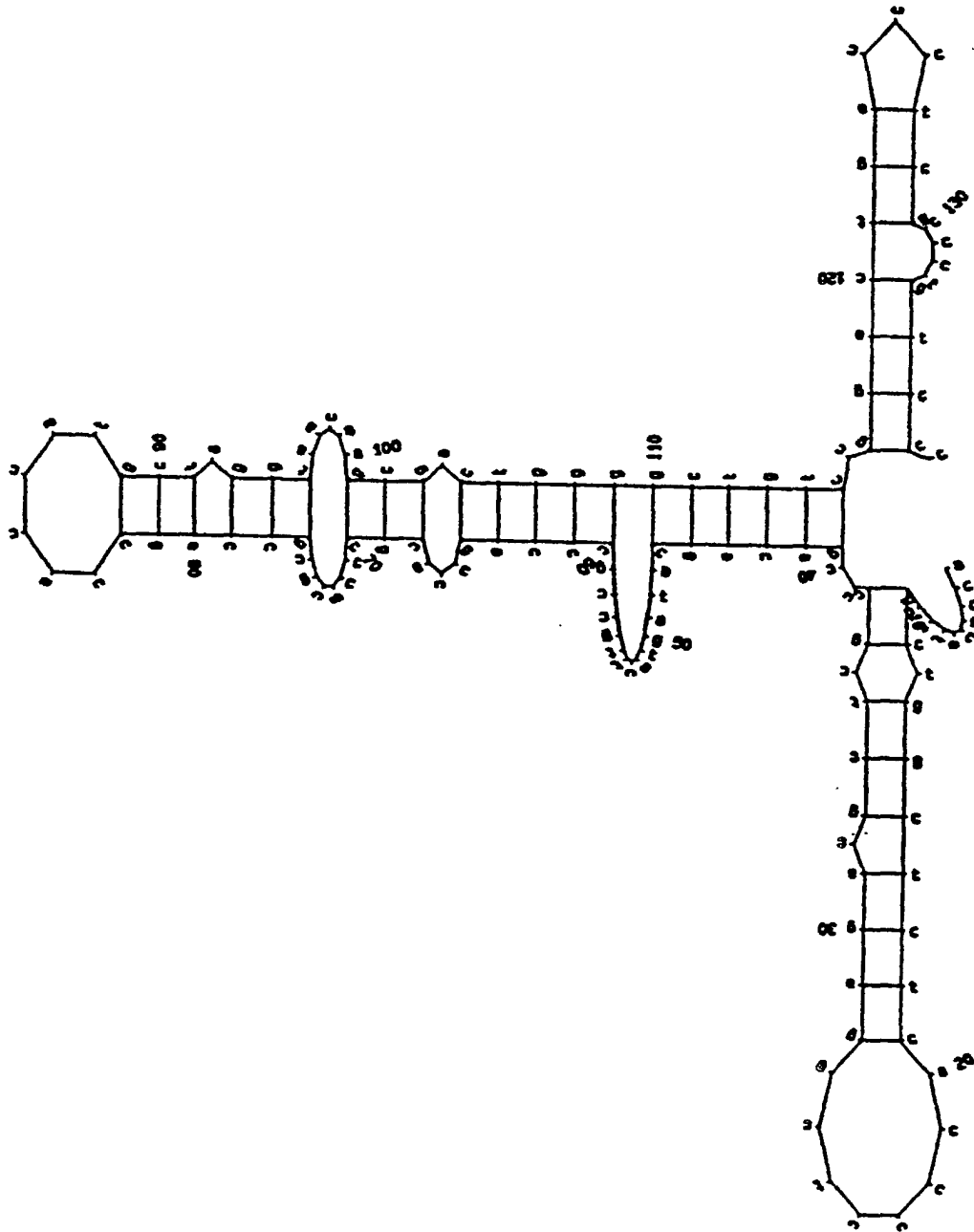
Scheme 1

Predicted secondary structure of preproNPY mRNA. The Scheme shows the predicted structure of the 5' end (1 to 138 bases) of the full preproNPY mRNA sequence published in GenBank Accession No. K01911. The secondary structure was predicted by using the MFOLD program of the Genetics Computer Group of the University of Wisconsin.

Squiggle plot of: osa1.mfold February 7, 19100 12:46

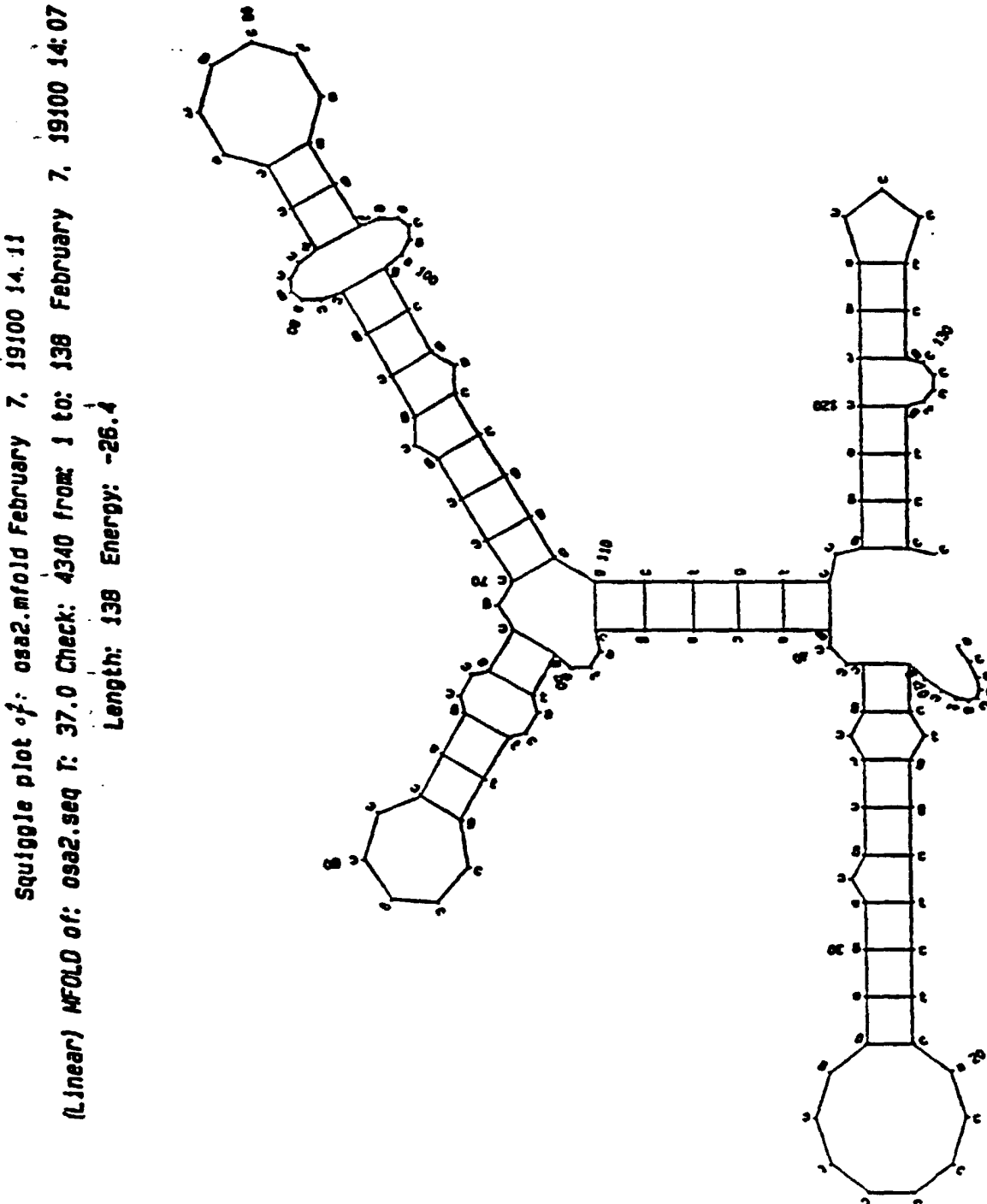
(Linear) MFOLD of: osa1.seq T: 37.0 Check: 5173 from: 1 to: 138 February 7, 19100 12:43

Length: 138 Energy: -28.4



Scheme 2

Predicted secondary structure of preproNPY mRNA. The Scheme shows the predicted structure of the 5' end (1 to 138 bases) of the full preproNPY mRNA sequence published in GenBank Accession No. K01911. The secondary structure was predicted by using the MFOLD program of the Genetics Computer Group of the University of Wisconsin. The mutated base T to C is base number 106.



Influence of the mutated NPY sequence on the function of NPY gene can be investigated in transgenic animals. A transgenic animal can be generated using targeted homologous recombination methodology. Both normal and mutated sequence of human NPY signal peptide (or any DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) or part thereof encoding the amino acid sequence of the mature mouse or human mature NPY peptide, where either i) the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline or ii) the leucine amino acid in position 7 of the signal peptide part of said preproNPY is unchanged) will be introduced into the sequence of NPY gene to replace the endogenous signal peptide sequence. Under these conditions, the endogenous NPY gene functions otherwise normally, but the synthesis of the preproNPY is regulated by either normal or mutated human NPY signal peptide sequence. This transgenic model can be used to investigate in a very specific manner the physiological importance of the mutated NPY gene. It also will provide an ideal preclinical model to investigate and screen new drug molecules, which are designed to modify the influence of the mutated NPY gene.

The invention is described more in detail in the following experiments.

EXPERIMENTAL

Materials and methods

Study subjects

The study population consisted of the participants of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD), a population-based epidemiologic study that was launched in the 1980's to investigate previously unestablished

risk factors for myocardial infarction, progression of atherosclerosis, and other major health outcomes in middle-aged men [Salonen, 1988; Lakka et al., 1994]. The study protocol has been approved by the Research Ethics Committee of the University of Kuopio, and all participants gave a written
5 informed consent to participate in KIID.

The total sample of the KIID study consists of 2,682 men who were recruited in two cohorts. The present study is based on the second cohort, which is an age-stratified sample of 42-, 48, 54-, and 60 year-old men
10 (N=1,516, participation rate 82.6 %) enrolled in the study between 1986 and 1989. A DNA sample was obtained for 1,137 men who were free from coronary heart disease at baseline.

Assessment of alcohol consumption

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A self-report quantity-frequency questionnaire [Kauhanen et al., 1997a; Kauhanen et al., 1997b] was used to record the level of alcohol use. The average weekly consumption of alcohol in pure ethanol (grams/week) was calculated based on the known alcoholic content of each beverage type and
20 the reported doses and frequencies of drinking sessions. We further calculated the proportion of heavy users consisting of those whose average daily consumption exceeded 3 standard doses (>230 grams of ethanol/week). One dose is a 12 fl ounce bottle of beer, 12 cl of wine, or a 4 cl shot of hard liquor. Serum gamma-glutamyltranspeptidase (GGT) and mean corpuscular
25 volume (MCV) were determined from baseline blood samples as biomarkers of excessive alcohol use. These biochemical measures were checked to see if any of the genotype groups showed biochemical signs of actual alcohol abuse.

Men who told they had not been drinking at all for at least 12 months were determined as abstainers (N=123, a total 12.1 %). Since abstainers are a heterogenous group consisting of those who have quit because of health problems, they were excluded from final analyses.

5

Covariates

A number of sociodemographic, behavioral and medical characteristics were assessed according the KIID protocol as described earlier [Salonen, 1988; 10 Lakka et al., 1994; Kauhanen et al., 1997a]. Age, place of living (urban/rural), marital status, educational level, current income, history of smoking in cigarette-years, and history of diagnosed chronic diseases and conditions (ischemic heart disease, diabetes, stroke, cancer, liver disease, 15 mental disorder) and history of trauma were recorded by a questionnaire and double-checked in the clinical interview. The data were used to examine the possible effect of confounding in the observed relationship.

Genotype analysis

20 PreproNPY genotype was determined by restriction fragment length polymorphism (RFLP) analysis from DNA extracted from the subjects' peripheral blood by an investigator unaware of phenotype. Briefly, the polymorphism appears as a thymidine(1128) to cytosine(1128) substitution generating a Bsi EI restriction site, which was used to genotype the subjects 25 for the Leu7Pro polymorphism, as described previously [Karvonen et al., 1998]. The PCR products were digested by Bsi EI [New England Biolabs, Inc. Beverly, MA, USA] and digestions were analyzed by electrophoresis on 2% agarose gel.

30 Statistical analyses

The allelic frequency distribution was tested for Hardy-Weinberg equilibrium by the X^2 -test. Statistical differences in the mean weekly alcohol consumption between the genotype groups were examined in the analysis of variance. Age and other covariates were adjusted for in analysis of covariance. The proportion of heavy drinkers in the genotype groups was compared using a chi-square test. P-values less than 0.05 obtained from the statistical tests were interpreted as statistically significant. Statistical computations were performed using the SPSS software for IBP RS/6000 [SPSS for Unix, SPSS Inc., Chicago, USA].

Results

The analysis of the Leu(7)-to Pro(7) polymorphism in the signal peptide part of the pre-pro-NPY and complete information on alcohol use was available for 889 alcohol using men. Of these, 790 (88.9 %) were genotyped as Leu(7)/Leu(7) homozygous, a total of 95 (10.7 %) were Leu(7)/Pro(7) heterozygous, and 4 (0.4 %) were Pro(7)/Pro(7) homozygous. The allele frequencies were 94.2 % (Leu) and 5.8 % (Pro). All men carrying either one or two Pro(7) alleles were pooled for further analyses. The study population was in Hardy-Weinberg equilibrium ($\chi^2=0.585$, 1 d.f., $p=0.44$).

Table I shows sociodemographic and behavioral background characteristics, and the proportion of men with diagnosed diseases in the two NPY genotype groups. There were no differences in the serum level of gamma glutamyl transpeptidase (GGT) or mean corpuscular volume (MCV) between genotypes. The means and standard deviations of GGT were 29.0 U/l (SD 29.4) among Leu(7)/Leu(7) homozygotes and 29.7 U/l (26.0) among those with Pro(7) ($p=0.83$). For MCV the means and standard deviations were 92.0 fl (SD 4.52) and 92.0 fl (SD 4.0), respectively ($p=0.93$).

The alcohol consumption in grams of pure ethanol per week is presented in Table II. Both the unadjusted mean consumption and the covariate-adjusted consumption were significantly (33 percent) higher among men who were carriers of Pro(7). The proportion of heavy drinkers (men who reported drinking on average over 230 grams of ethanol/week or over 3 standard doses/day) was also higher among men with a Pro(7) substitution (13.1 % vs. 8.2 %) ($p=0.10$).

10 Table I. Means (standard deviations) and proportions of background variables by the NPY genotype.

	Leu(7) homozygotes (N=790)	Pro(7) carriers (N=99)
Age (years)	56.1 (SD 6.7)	56.1 (SD 6.9)
Living in rural area	21.8 %	27.0 %
Annual income (US \$)	24,130 (SD 15,918)	26,862 (SD 14,771)
Educational level (1= low, 7= high)	2.05 (SD 1.75)	2.13 (SD 1.92)
Married	87.1 %	86.9 %
Cigarette smoking (pack-years)	141.3 (SD 292.1)	147.4 (SD 311.7)
Ischemic heart disease	21.1 %	13.1 %
Diabetes	5.6 %	5.1 %
History of cancer	2.4 %	5.1 %
History of stroke	2.6 %	1.0 %
Liver disease	0.4 %	1.0 %
History of mental disorder	4.6 %	6.1 %
History of trauma	10.4 %	10.2 %

Table II. Mean weekly alcohol consumption in pure ethanol according to the NPY genotype.

5

			P-value
	Leu (7) homozygotes (N=790)	Pro(7) carriers (N=99)	
Unadjusted mean alcohol consumption (g/wk)	86.3 (SD 127.6)	115.0 (SD 173.9)	0.030
Mean alcohol consumption (g/wk) adjusted for all covariates*	86.4	114.7	0.035

*Adjusted for age, place of living, education, income, marital status, smoking history in cigarette-years, history of ischemic heart disease, diabetes, cancer, stroke, liver disease, mental disorder and trauma.

10

Discussion

We observed an increased alcohol consumption in a population sample of middle-aged men who were homozygous or heterozygous for the variant allele in a common polymorphism substituting Leu(7) by Pro(7) in the signal peptide part of neuropeptide Y (NPY). Presence of Pro(7) was associated with approximately one-third (33 %) higher average consumption of ethanol as compared to homozygous subjects with the Leu(7)/Leu(7) genotype. The proportion of heavy consumers who report using over 230 grams of ethanol/week was also higher among men with Pro(7) mutation, although this difference did not reach statistical significance due to smaller numbers of subjects.

Our study is the first one to show a relationship between a common NPY polymorphism and alcohol use in humans. The results are in line with the findings from a number of recent animal studies [Ehlers et al., 1998a; Ehlers et al., 1998b; Thiele et al., 1998; Cockerill, 1998; Tecott and Heberlien, 5 1998] that have shown an inverse relationship between levels of NPY in central nervous system and preference for alcohol. Mice with no neuropeptide Y are especially fond of alcohol and less sensitive to the effects of ethanol as compared to mice that have normal or extra neuropeptide Y levels [Thiele et al., 1998], and alcohol-preferring rats have lower levels of 10 NPY in amygdala, hippocampus, and frontal cortex [Ehlers et al., 1998a].

The allele frequencies in our study were close to those seen earlier in two Finnish populations [Karvonen et al., 1998]. It is highly unlikely that the observed association could be due to a stratification error in sampling, or 15 population admixture, since Finns are known to be genetically a rather homogenous population.

Many sociodemographic factors are known determinants of alcohol use. In our study the social background among men with and without Pro(7) was 20 similar. The two groups were of the same age and had similar educational background. Slightly more men with Pro(7) were living in rural communities, and this group also had a little higher average income. Smoking history was similar in both groups. It was somewhat unexpected to observe a higher prevalence of ischemic heart disease history among the Leu (7) 25 homozygotes, since earlier findings have shown this genotype to associate with lower serum levels of total and LDL cholesterol [Karvonen et al., 1998]. Adjustment for all these variables in the multivariate model did not affect the observed association between the NPY polymorphism and alcohol consumption, indicating that these variables did not confound the findings.

There are several physiologically plausible mechanisms that can explain the effect of NPY on alcohol use. NPY is an inhibitory neuromodulator that acts widely in the brain. The NPY receptors couple to heterotrimeric G proteins that inhibit production of cyclicAMP [Thiele et al., 1998; Lamme, 1995], so
5 it is possible that NPY inhibits cAMP production in response to alcohol, thus limiting alcohol intake. Central administration of NPY reduces anxiety, and NPY-deficient mice score high on measures of anxiety [Heilig et al., 1992; Palmiter et al., 1998]. The development of alcohol preference may in part depend on the relative lack of tension-reducing NPY.

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Chronic exposure to ethanol in rats affects NPY levels in hypothalamus in a fashion similar to food restriction [Ehlers et al., 1998a]. NPY has an important role in the hypothalamic regulation of energy balance by potently stimulating short-term food intake [Clark et al., 1985; Levine and Morley,
15 1985; Stanley and Leibowitz, 1985]. Centrally administered NPY also increases the expression of lipoprotein lipase mRNA and enhances the enzyme activity in white fat favoring lipid storage [Billington et al., 1991; Billington et al., 1994]. Thus, NPY might unspecifically affect the consummatory behaviors regarding both food intake and alcohol drinking.
20 However, there is a lack of NPY transgene expression in the arcuate nucleus of the hypothalamus, a region thought to regulate food intake [Thiele et al., 1998; Palmiter et al., 1998]. This indicates that the effects of NPY on alcohol use are probably not mediated through similar mechanisms as with food and calorie intake.

25

To our knowledge, there is only one earlier human study examining the possible relationship between neuropeptide Y and addictions. Roy and coworkers [1990] did not observe significant differences of cerebrospinal fluid (CSF) levels of NPY between male alcoholics and normal controls.

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Alcoholics, however, do not represent the population at large. It is also

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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